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Research Paper

Lnc-PCDH9-13:1 Is a Hypersensitive and Specific Biomarker for Early Hepatocellular Carcinoma

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ABSTRACT

Background: Long non-coding RNAs (lncRNAs) show great potential as diagnostic tools in many diseases. We aimed to develop sensitive and noninvasive biomarkers in saliva for detecting early hepatocellular carcinoma (HCC).

Methods: Candidate lncRNA biomarkers identified by Agilent microarray were subjected to validation using qPCR for the quantification of their expression levels in independent tissue, plasma and saliva sample sets, including healthy controls, HBsAg carriers, patients with chronic Hepatitis B, liver cirrhosis, early HCC, and advanced HCC. Levels of candidate biomarkers were also measured in totally 108 saliva samples from patients with any one of other nine leading causes of cancer death in men and women.

Findings: Lnc-PCDH9-13:1 was significantly elevated in HCC tissues, plasma and saliva of HCC patients compared with healthy controls and groups of several benign liver diseases and other leading cancers. Its level was significantly reduced after curative hepatectomy but significantly elevated again if HCC recurrence occurred. Salivary lnc-PCDH9-13:1 showed reasonable specificities and sensitivities for detecting HCC compared with several control groups. Furthermore, the overexpression of lnc-PCDH9-13:1 promotes cell proliferation and migration in vitro.

Interpretation: Salivary lnc-PCDH9-13:1 is a desirable biomarker for early HCC. It may help warrant prospective validation with larger sample sizes in multi-centers.

1. Introduction

Liver cancer, over 90% of which is hepatocellular carcinoma (HCC) categorized by pathology, is the second leading causes of cancer death worldwide in men and the sixth in women, with an overall 5-year survival rate of 5%–9%. An estimated 782,500 new liver cancer cases and 745,500 deaths occurred worldwide during 2012, with China alone accounting for about 50% of the total new cases and deaths [1]. In the USA, incidence rates continue to increase rapidly for liver cancer with totally 42,220 new cases and 30,200 deaths expected to occur in 2018 [2]. In England, the incidence of liver cancer was higher in all ethnic groups compared to Whites, with the highest rates seen in Chinese (four times higher) [3]. Thus, liver cancer is one of the most fatal cancers in China, the USA, and the world to date. Curative hepatectomy can improve the 5-year survival rate to 69%, but unfortunately, approximately 80% of patients with HCC are untreatable because of advanced tumor stages at presentation [4, 6, 30]. Hepatitis B virus (HBV) which infects approximately 350 million people worldwide, is the major risk factor of HCC [7] and it accounts for up to 70% of all HCC patients [8]. Patients with chronic hepatitis B develop cirrhosis at a rate of approximately 2%–10% per year, leading to HCC in 80% to 90% of individuals finally [9]. Over 5% of the populations in Asia and sub-Saharan Africa chronically infected with the virus. Accordingly, early diagnosis and surgery are vital for treating HCC.

Studies suggest that long non-coding RNAs (lncRNAs) (≥200 bp) in HCC tissues and blood of patients with HCC show good diagnostic accuracy in detecting HCC [10]. Tissues may secrete lncRNAs into the blood by necrosis and apoptosis. Owing to the extensive blood supply in salivary glands, saliva is considered a terminal product of blood circulation. Saliva may include many components that are derived from blood, because salivary acinar cells produce saliva using blood materials. Hence, saliva can play diagnostic roles in various diseases [11].

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We aimed to identify novel and noninvasive lncRNA biomarkers in saliva to help diagnose HCC.

2. Materials and Methods

2.1. Patients and Samples

The study was conducted mainly according to the updated Standards for Reporting of Diagnostic Accuracy (STARD) 2015 reporting guideline for diagnostic accuracy studies [12]. The Healthy controls were defined as individuals with negative results by health examinations, including chest X-rays, oral examinations, abdominal ultrasounds, faecal occult-blood testing, blood cancer biomarker assays (AFP, CEA, CA19–9), HBV antigen, HCV, HIV, and syphilis antibodies. Inactive HBsAg carriers (IHC) had persistent HBV infection of the liver (positive HBsAg over 6 months with serum HBV DNA $<10^5$ copies/ml) and no laboratory indications of ongoing necroinflammatory hepatic functions: persistently normal aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations. Chronic hepatitis B (CHB) was defined as chronic necroinflammatory liver function caused by persistent HBV infection (positive HBsAg over 6 months with serum HBV DNA $>10^5$ copies/ml and persistent or intermittent elevation in AST or ALT concentrations). Patients with liver cirrhosis (LC) were confirmed by biopsy or two imaging modalities (hepatic ultrasound with CT or MRI) [13]. Terminal HCC (Stage IV) was diagnosed based on biopsy of the tumor or CT/MRI [14]. The histopathology results of the patients with the HCC (Stages I, II, III), lung, stomach, colon & rectum, prostate, esophagus, pancreas, leukemia, urinary bladder, non-Hodgkin lymphoma, breast, cervix uteri, ovary cancers, which were 10 leading causes of cancer death in men and women worldwide nowadays [1], were confirmed by pathology after surgical tumor resection or biopsy. Patients with other cancers and benign liver diseases were evaluated with regard to marker specificity. Normal human liver tissues were obtained from distal normal liver tissues of liver hemangioma. The normal liver tissues from liver hemangioma were also determined by pathology. No concurrent oral, infectious, autoimmune diseases and diabetes mellitus were diagnosed in all participants. All HCC patients were positive with HBsAg. Cancer patients with a diagnosis of concurrent two cancers and those undergoing chemotherapy and radiotherapy prior to sample collection were also excluded. Cancers were staged according to The American Joint Committee on Cancer: 8th Edition on Cancer Staging [15]. 100 saliva samples was obtained from 50 patients with early HCC over 1 week before and after curative surgery, and saliva from 6 HCC patients among the 50 patients with early HCC was available at the time of documented HCC recurrence with the use of radiographic imaging and, usually, pathological confirmation of recurrence (Fig. 1). Our previous study show adjacent HCC tissues were infiltrated with inflammatory cells, cytokines and cirrhosis [16]. Hence, the majority of adjacent HCC tissues were not "normal" liver tissues. So the levels of lncRNAs in adjacent HCC tissues were not measured and compared since plasma and saliva levels of the candidate lncRNA biomarkers were compared with those of normal healthy controls. The tissue, plasma and saliva samples from all participants were collected consecutively and retrospectively if they met the inclusion criterion between April 2011 and August 2016 at The Third Affiliated Hospital of Sun Yat-sen University.

Institutional review boards or ethics committees from our hospital approved the study protocol. All participants provided written informed consent for their information to be stored in the hospital database and used for research.

2.2. Procedures

Saliva samples were collected as previously described [17]. After the tissue, plasma and saliva samples were collected and stored in the −80 °C lab freezers, the following procedures were finished with seven days. Total RNAs were extracted from frozen liver tissues using TRIzol (Thermo Fisher Scientific, USA), and total RNAs in 1.2 ml of plasma or saliva were isolated by the mirVana PARIS Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocols. The lncRNA profiles of the three HCC liver tissues and three normal liver tissues assayed using Agilent Technologies, USA. The measurements of lncRNAs by quantitative polymerase chain reaction (qPCR) were performed as previously described [18]. Each qPCR reaction contained negative controls included no template control, no reverse transcriptase control, and no amplification control. All reactions including controls were carried out in triplicate. Ct values >35 were excluded from the analyses. The expression levels of each lncRNA were normalized to that of *β-actin*. All expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The indeterminate and missing data were excluded, and qualified samples would be made up the original sample sizes. The expression of candidate lncRNAs in paraffin sections was detected in situ hybridization (ISH) according to the manufacturer's instructions of the probes (Exiqon, Denmark). The expression of APF in paraffin sections was assayed by immunohistochemistry (IHC) with primary antibody purchased from Gene Company, Hong Kong. Cells with clear brown staining were regarded as positive cells. The APF levels were calculated by enzyme-linked immunosorbent assay (ELISA) with antibody obtained from Cloud-Clone Corp, USA. All experiments were performed at least three times on different days and in triplicate. The sequences of each primer and probe, and detailed

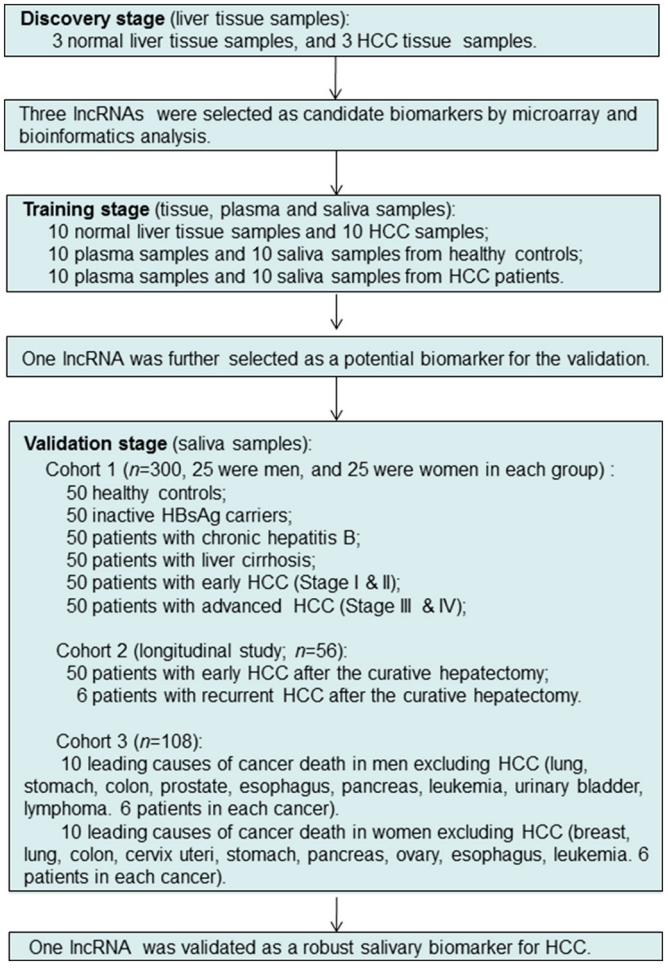

Fig. 1. Study design. Our study consisted of three stages. Candidate lncRNA biomarkers were selected by microarray and bioinformatics analysis. The levels of the selected candidate biomarkers in tissue, plasma and saliva samples were measured by qPCR and analyzed by biostatistics. The diagnostic performances of the selected lncRNAs were validated in three cohorts.